

Observations on Electron Micrographs of DNA

Verkürzte Fassung der
INAUGURALDISSERTATION

zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

JÖRG RICHTER
aus Darmstadt (Deutschland)

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag der Herren Prof. Dr. MAX THÜRKAUF und PD Dr. ARMIN
RAMEL.

Basel, den 1. Juni 1965

Prof. Dr. H. ANNAHEIM
Dekan

Herrn JÖRG RICHTER wurde von der Philosophisch-Naturwissen-
schaftlichen Fakultät der Universität Basel am 1. Juni 1965 erlaubt,
diese verkürzte Fassung seiner am 1. Juni 1965 genehmigten Inaugural-
dissertation drucken zu lassen. Die vollinhaltliche Arbeit mit dem Titel:

*«Elektronenmikroskopische Untersuchungen an Kalbsthymus-
Desoxyribonukleinsäure»*

kann eingesehen oder entliehen werden:

1. im Physikalisch-Chemischen Institut der Universität Basel;
2. in der Universitätsbibliothek Basel;
3. in der Schweizerischen Landesbibliothek in Bern.





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OBSERVATIONS ON ELECTRON MICROGRAPHS OF DNA

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(Received November 12th, 1965)

SUMMARY

Calf-thymus DNA in diluted solutions (10^{-3} – 10^{-9} g/ml) of different ionic strength (0–1 M ammonium bicarbonate, pH approx. 7) was subjected to electron-microscopic investigation. A special freeze-drying method was applied for fixation, and the contrasting was performed at the temperature of liquid nitrogen by means of Pt–C coating. Denatured, degraded and chymotrypsin-incubated samples were prepared as well.

Contrary to expectations, no single threads could be seen on the electron micrographs. The DNA emerged from these experiments as a highly crosslinked network. The likelihood of preformed crosslinks is discussed in the light of present knowledge favouring such a structure. An attempt is made to reconcile the present results with the current picture of DNA in solution obtained from light-scattering and hydrodynamic methods. Some doubts are raised regarding the correct interpretation of these indirect measurements.

INTRODUCTION

The recent work of KLEINSCHMIDT *et al.*¹ considerably intensified electron microscopic studies of DNA. A great number of attempts to reveal structural details of DNA in solution by means of electron microscopy are of earlier date, however. From experiments with native sonicated DNA, HALL AND DOTY² were able to evaluate the most probable molecular weight distribution. However, in the case of unfragmented samples, with HALL's technique³ no reliable information could be gained. With highly diluted, just as with concentrated solutions, reticulations were observed, but they were interpreted in different, partly even in contradictory ways^{4,5}. When denatured DNA was prepared according to the procedure of HALL, flattened patches or "puddles" or "blobs" were seen on the electron micrographs^{5–7}.

Very long unbroken single threads were observed by BEER⁸. In his procedure, a membrane with weakly basic ion-exchange properties is streaked along the surface of a DNA solution of low ionic strength and well defined pH. A similar transfer

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technique together with subsequent positive staining, rather than the usual shadow-casting, was applied by STRUČKOV⁹. The material utilized was rat-thymus DNA, carefully extracted by the phenol method. The high degree of polymerization which is preserved in this procedure could obviously be the reason for the network of high density seen on these electron micrographs. The protein content of his DNA was 3 %!

The electron micrographs of KLEINSCHMIDT *et al.*¹ are very well known today. In his experiments the DNA was liberated from phage heads through osmotic shock treatment. The appearance of the DNA on his pictures has a resemblance to untwined coils on which a higher degree of order is superimposed. Free ends are rarely seen in his pictures.

Each of the methods mentioned above has its serious shortcomings which arise either from its inherent tendency to produce artefacts, especially by the high shear gradients mostly employed, or from its selection characteristics. Freeze-drying could be a means to reduce artefacts¹⁰. However, extreme care must be taken during the following contrasting to secure conditions which stabilize the structure.

Along this line of thought a method was developed in this Institute¹¹ which allows the object to cool down to the temperature of liquid nitrogen; it is shadow-cast at this temperature with platinum-carbon without interrupting the high vacuum. The well-known coating effect due to diffusing carbon particles along the surface of the material provides the required additional stability.

In this paper a few electron micrographs of calf-thymus DNA of very low protein content are presented which were obtained with this new procedure. The most important result of these experiments is the highly crosslinked appearance of the DNA, a result which is essentially independent of the pretreatment given to the solution. The "cleanest" pictures were obtained, however, when the DNA was prepared from salt-free solutions. More tentative are the experiments where the DNA was either heated, subjected to high shear gradients, or incubated with chymotrypsin.

MATERIALS AND METHODS

DNA solutions

Calf-thymus DNA. Calf-thymus DNA (Na-salt), prepared according to different methods, was kindly donated by Dr. M. H. F. WILKINS, Dr. J. POUYET and Dr. H. P. VON HAHN. The residual protein content was indicated to be 0.2–0.3 %. Occasionally also salmon-sperm DNA (Calbiochem Lot 40 109) with arginine <0.1% was used. It gave the same results.

Stock solutions. Stock solutions were prepared by dissolving 1 mg DNA/ml in bidistilled water at 5° without stirring. The solution time was approx. 15 h. No changes, neither by means of ultraviolet spectroscopy and viscosimetry nor by electron microscopy itself, could be detected in such a solution after several weeks. No influence on the result were observed, either, when the stock solution was centrifuged for 1 h at approx. 18 000 $\times g$. For this reason centrifugation was not performed routinely. All operations with salt-free DNA solutions were carried out in the cold to avoid denaturation.

Final solutions. Final solutions were prepared by diluting the stock solution

with distilled water or with ammonium bicarbonate buffers of various ionic strengths to the desired end value between 10^{-3} and 10^{-9} g/ml. The pH of the buffer was adjusted initially to 6.5.

Pretreated solutions. In order to induce thermal *denaturation*, highly diluted completely or nearly salt-free solutions were warmed to 45° for a few hours. The increase of the extinction coefficient at $260\text{ m}\mu$ amounted in all these cases to approx. 30 %. *Degradation* was performed by high shear forces ($G \cong 10^5\text{ s}^{-1}$) or by sonication. *Proteolysis:* The highly diluted cold solution of DNA in buffer of 10^{-3} – 10^{-4} M and pH 8 was made 0.01 % in chymotrypsin. Incubation time was approx. 10 h.

Specimen preparation and electron microscopy

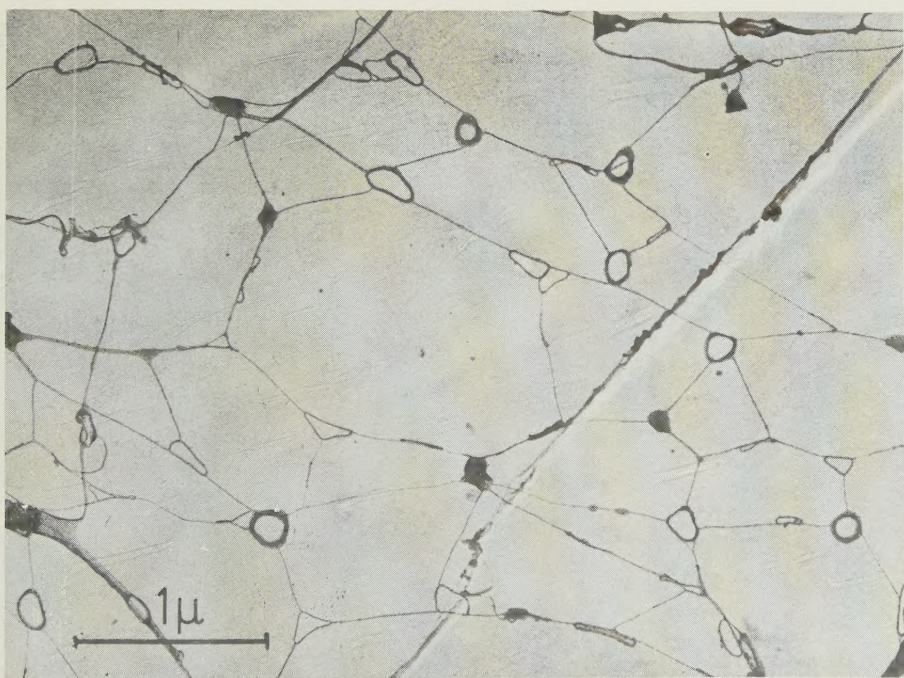
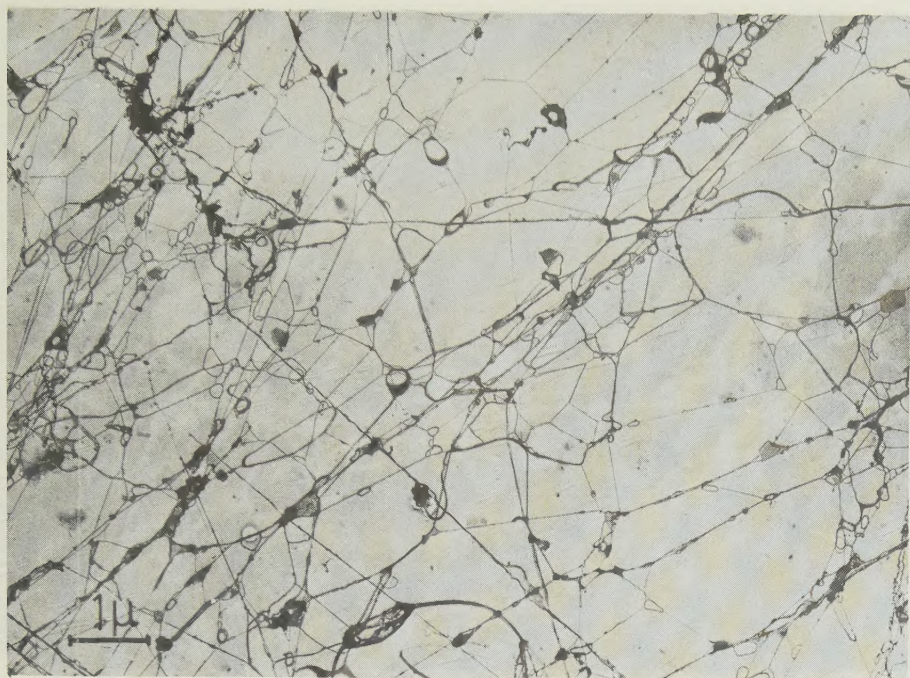
Before transfer the solutions were regularly shaken for a few seconds. Because of the inherent high shear gradients, spraying was used only for solutions of high salt content. The most successful transfer procedure was the following: the formvar-coated grid was submerged vertically into the DNA solution, pulled out vertically and dried at the rear side with filter paper. Immediately afterwards the grid was dipped into liquid nitrogen.

The details of the freeze-drying apparatus used in the special procedure are described elsewhere¹¹. The samples were kept at a very low temperature during the evacuation process of the machinery and were thereafter, in one step, (a) freeze-dried for 1–10 h at -30° to -120° and 5×10^{-6} mm Hg (b) without change of the high vacuum, brought to -180° with liquid nitrogen, and then (c) shadow-cast with Pt–C.

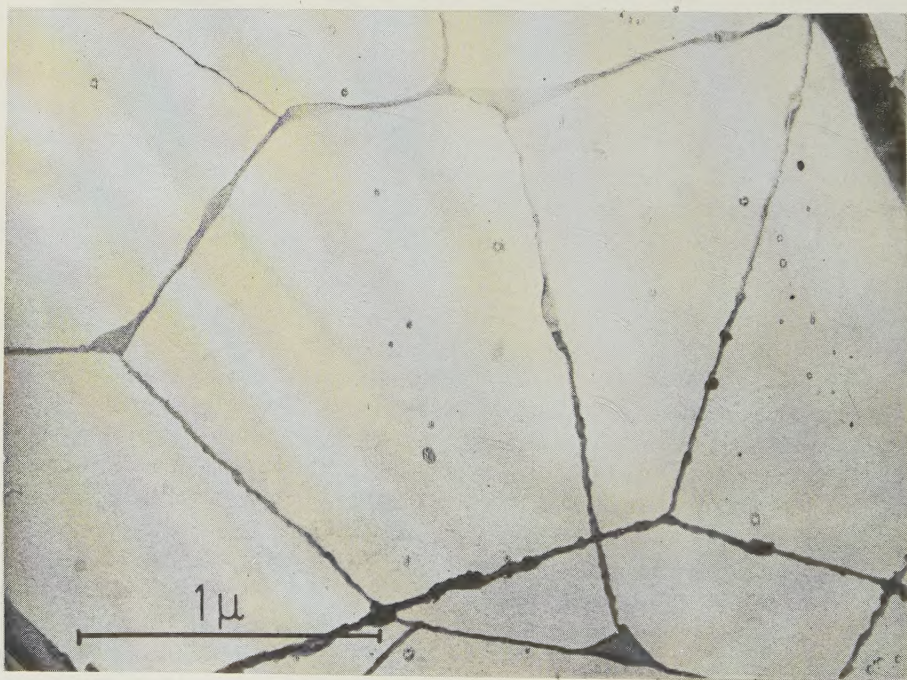
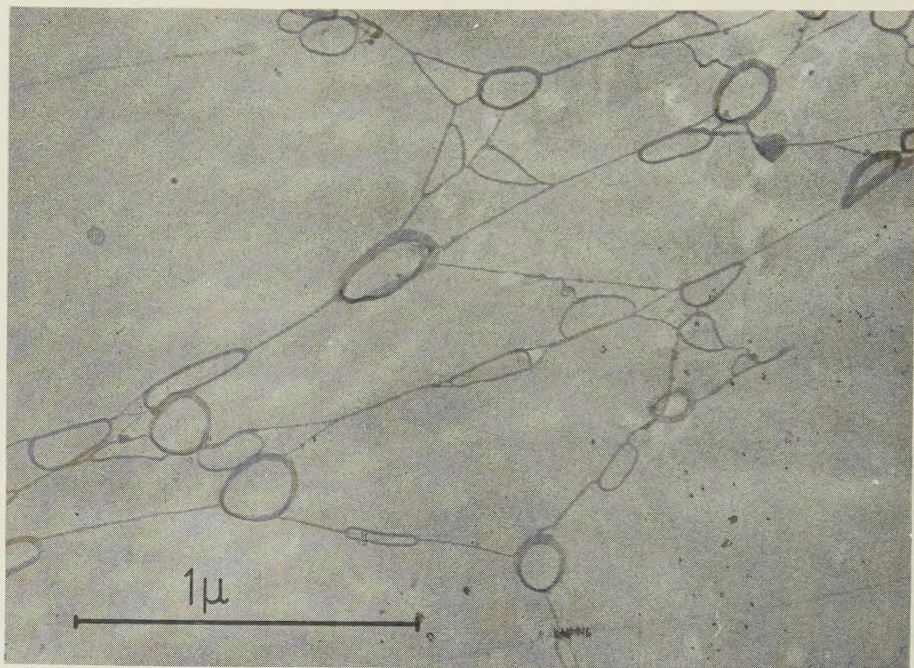
Measurements by means of a thermocouple near the object showed that the rise of temperature during shadow-casting was imperceptible. The DNA-threads became coated with carbon. Hence, their mechanical stability was considerably increased. Mostly the evaporated Pt–C layers were $40 \pm 10\text{ \AA}$ thick. The majority of the pictures presented in this paper was obtained with a Siemens Elmiskop I which was routinely calibrated with a carbon grating replica ($0.883\text{ }\mu$) supplied by Fullam, Schenectady, N.Y.. Each picture is photographically magnified about two times.

RESULTS

Micrographs 1–10 are representative of a series of more than 50 experiments. The network structure well-developed on each of these pictures is typical for the whole series. Shadows can only be seen if the material reached a height above the foil of less than a few microns. Otherwise shadows are diffuse due to scattered platinum particles. It can be seen that DNA can be visualized best by means of this special procedure if the DNA was dissolved in pure water (Figs. 1–5). The presence of salt led to side effects which gave a blurred appearance (Fig. 6). This was always the case if the electrolytes were abundant relative to the DNA. The pictures of all preparations with salt-containing solutions suggest a higher degree of strand aggregation compared with those from salt-free solutions (Figs. 6 and 7). When the solution was subjected to high shear gradients undefined patterns rather than the expected degradation of the DNA material were observed.



Figs. 1 and 2. Calf-thymus DNA, freeze-dried from salt-free solution and Pt-C shadowed at an object temperature of -180° . The angle was 60° . The concentration of DNA was $5 \cdot 10^{-6}$ g/ml.



Figs. 3 and 4. Calf-thymus DNA. Views from reproduction experiments to 1 and 2.



Fig. 5. Calf-thymus DNA from salt-free solutions at very low concentration (DNA 10^{-7} g/ml).

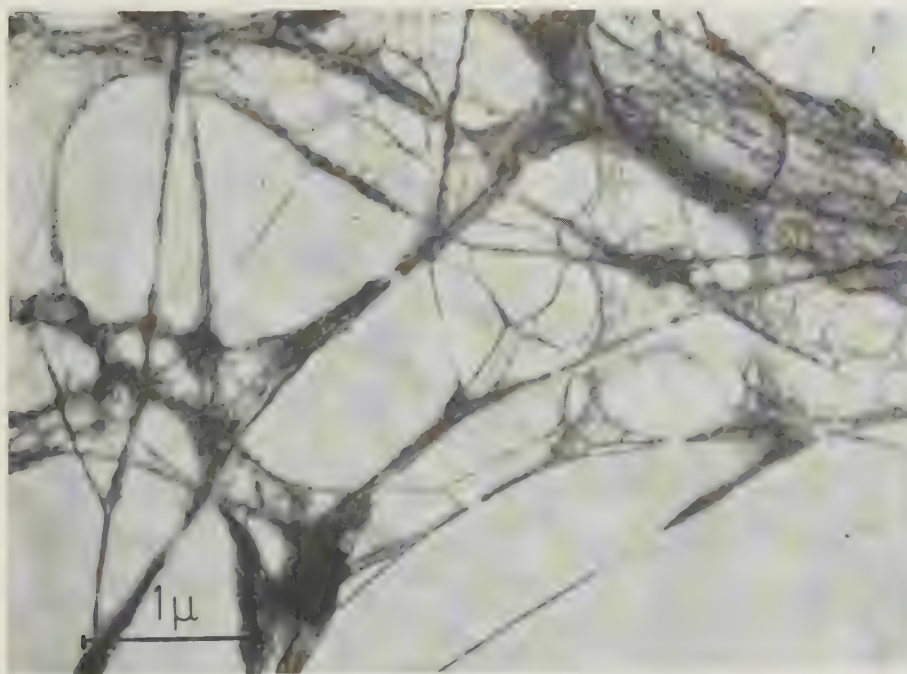


Fig. 6. Calf-thymus DNA, prepared as before. The solution contains 1 M ammonium bicarbonate. Concentration of DNA was $5 \cdot 10^{-5}$ g/ml. The picture shows the higher degree of aggregation.

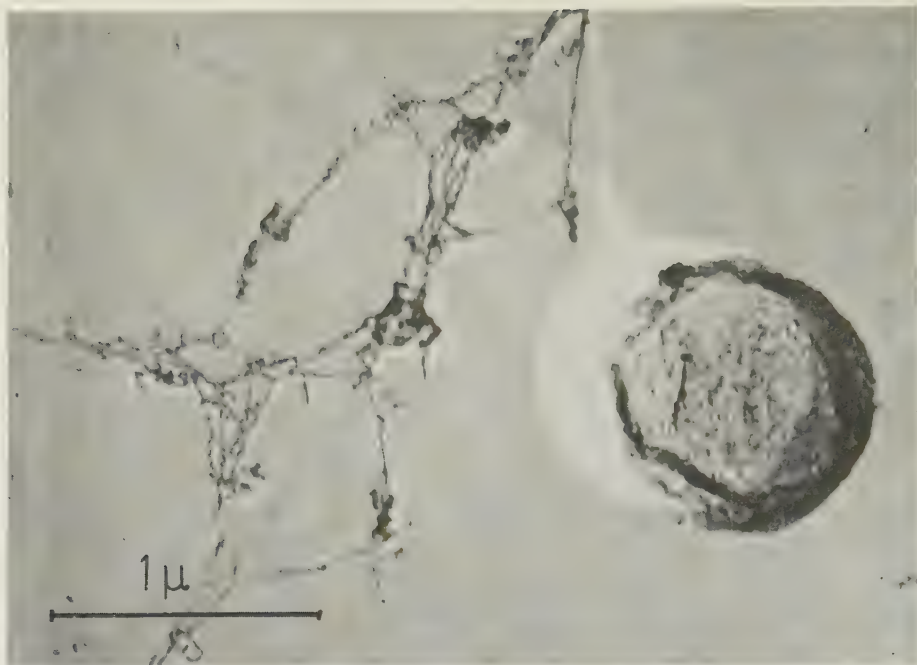


Fig. 7. Highly concentrated (10⁻³ g/ml) DNA solution, 0.2 M in ammonium bicarbonate, was sprayed onto a precooled grid. One can see adhering portions of crosslinked structures.

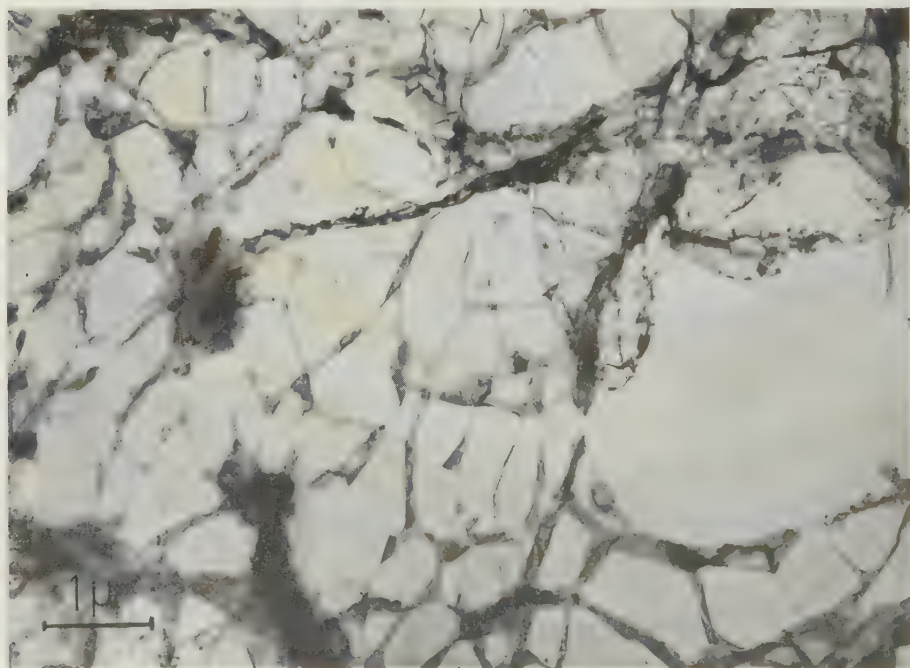


Fig. 8. The freeze-drying process stopped at an early state. A preformed network becomes visible.

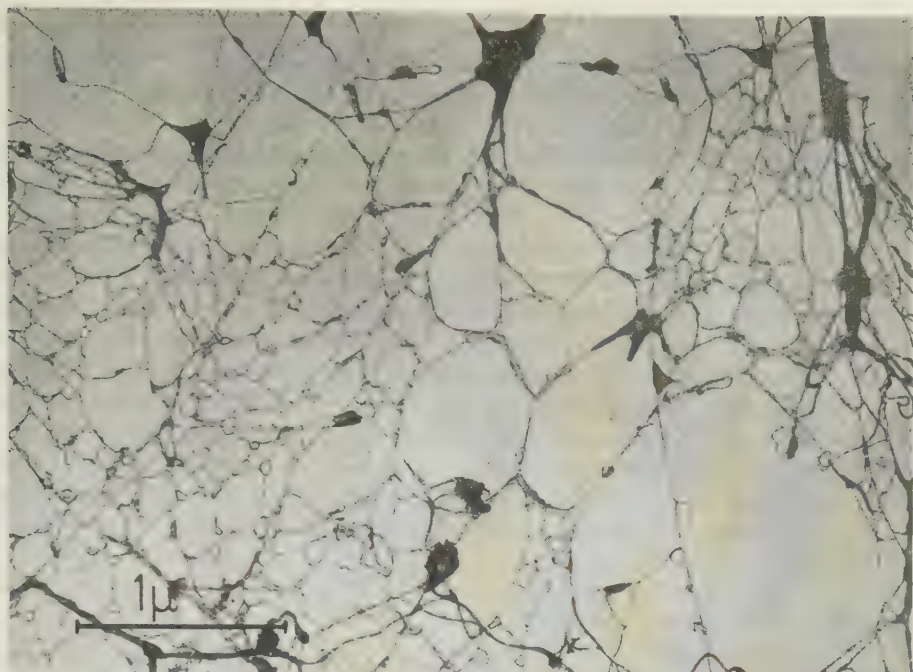


Fig. 9. Salt-free DNA solution, submitted to thermal denaturation before freeze-drying. The threads seem to have lost their smooth appearance.

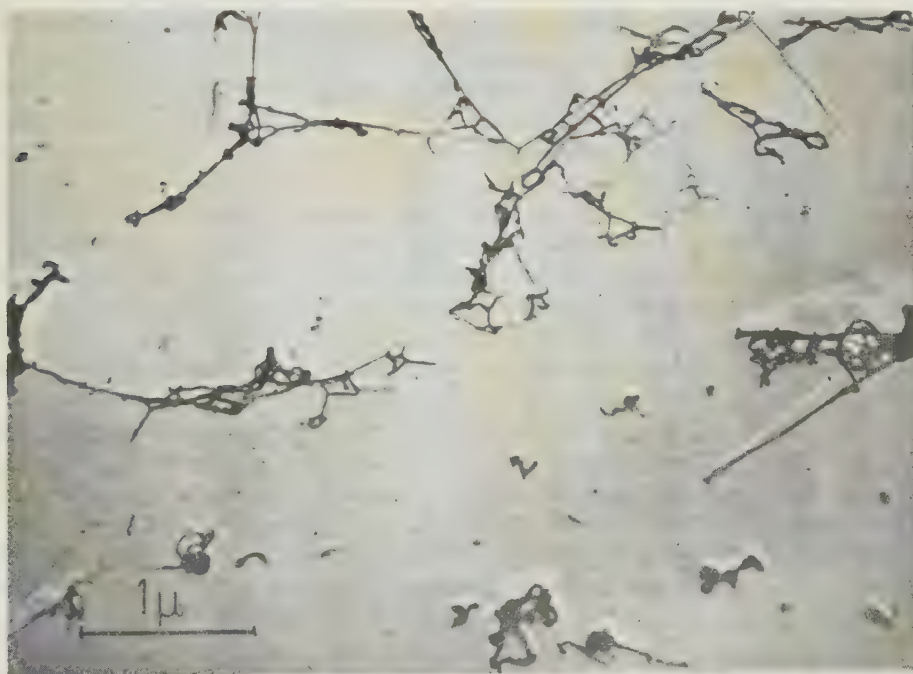


Fig. 10. Calf-thymus DNA solution treated with chymotrypsin. The network structure is partly destroyed.

In case of denatured DNA (Fig. 9) the threads seem to have lost their smooth appearance. This material was also very sensitive to the electron beam: the network collapsed into little blobs while the shadows of an intact network structure can still be recognized on the foil. Only in the case of DNA incubated with chymotrypsin and subjected to proteolysis could the expected influence be visualized: the network was to a great extent destroyed, and a great number of loose ends became visible (Fig. 10).

A very conspicuous characteristic present in nearly all micrographs with varying frequency and size are cyclic structures. It seems that they are a part of the network. It remains unclear, however, what kind of artefact — if any — might have caused their formation. On warming and shaking a moderately concentrated cool DNA solution one can always see small bubbles which are very stable. The smallest we have seen are about 1μ in diameter. The question is whether the rings are pictures of these bubbles. Since the rings in our plates have diameters down to 0.1μ , very high internal pressures would have existed if they had been bubbles. Adsorption of DNA to the surface, by which bubbles could be made visible, could change the surface tension so that the high stability of the bubbles becomes possible.

DISCUSSION

The most important finding of these experiments is the highly crosslinked appearance of the DNA after freeze-drying. The different appearance of the same material after incubation with chymotrypsin (Fig. 10) favours the conclusion that the crosslinked structure of DNA in solution is genuine and not merely an artefact due to freeze-drying. Changes in the physical properties of the DNA which may occur as a result of the incubation could influence the mode of aggregation which takes place, for instance, during the sublimation process; however, there are some arguments which speak against the explanation that all or most of the crosslinks are formed during the freezing or during, or even after, freeze-drying.

On dipping the small quantity of solution with the grid into liquid nitrogen it becomes solid within a few tenths of a second. Such high freeze velocities (approx. $-100^\circ/\text{sec}$) suffice to conserve such delicate structures as living systems of micro-organisms because the water grows solid amorphyously (vitrifies). Furthermore the freezing-front migrates about 10^4 times quicker than the whole DNA particle by diffusion, if one accepts a diffusion coefficient of $10^{-8}\text{ cm}^2/\text{sec}$. Considering this one must expect that the DNA strands would be built into the ice-matrix without essential change of their conformation. At least at the lowest freeze-drying temperature (approx. -120°) no recrystallization should occur. Quite generally one would rather expect crystallization and recrystallization to destroy complicated hyperstructure than to form it.

A number of ideas and observations argue against the formation of the network by the freeze-drying process. On the one hand a high mobility of the threads — unlikely at such low temperatures — is necessary to explain the connexion to neighbouring threads. On the other hand one wonders that free and coiled ends are so rarely seen. Even at extremely high dilutions (10^{-7} – 10^{-9} g/ml) many more crosslinked strands (with over-all length mostly $>10\mu$) are visible than single strands (Fig. 5).

Preparations in which the sublimation process of ice was not very advanced support the hypothesis of a preformed network-structure (Fig. 8) just as observations of rings and crossings do. If the rings had still enough ice around themselves, they appear cyclic. In the course of sublimation they follow the pulling of clinging threads and become triangular (Figs. 2 and 3). At many crossings and ramifications, one can see stretched membranes which cannot have resulted from placing the dry strands one upon another (Figs. 1, 2 and 4).

In spite of partial collapse, such a perceptibly intact and three-dimensional network could be understood only on the basis of its preformation, otherwise it would collapse completely onto the grid in a flat lattice during the drying process. Sprayed solutions with native undegraded DNA in our preparations show agglomerates to which portions of network very often adhere, torn with them because of the high shear forces applied (Fig. 7).

At variance with current views on the structure of DNA in solution we would like to discuss, at least for DNA from animal tissues, the possibility of network-structure. The ideas put forth in the following are developed in greater detail elsewhere¹².

Apart from electron microscopy, the main sources of information regarding the behaviour, size and shape of DNA in solution are measurements of light-scattering, sedimentation and viscosity behaviour. It is unfortunate, however, that only viscosity measurements have been made systematically in relation to the ionic strength of the solution. While diluted solutions of very low electrolyte content ($< 10^{-3}$ M) show high concentration effects in η_{spec}/c and S , measurements on solutions of moderate and physiological NaCl concentration suggest a tendency to aggregation of the polyion threads¹³. EIGNER AND DOTY¹⁴ reviewed $[\eta]$ and S° -coefficients and mol. wt. data from light-scattering measurements on DNA-solutions whose electrolyte content ranged between 0.1 and 1.0 M. Improving the relation given by DOTY *et al.*¹⁵ in 1958, they show that S° is uniquely related to $[\eta]$ over a wide range of mol. wt. They concluded therefore that DNA molecules must form a homologous series independent of their origin and preparation. It is probable, however, that in these solutions the properties of the individual DNA threads are suppressed due to aggregation.

Even the purest DNA samples contain an amount of residual protein which by means of the most rigorous methods cannot be reduced below a value of approx. 0.3 %. It is very possible that these most obstinate contaminations represent polypeptides or small proteins which are covalently bound to the DNA (ref. 16). Hence, one is inclined to assume that these proteins could be the cause of parallel aggregation or crosslinking. This assumption is supported by the changes of physical properties which accompany chymotrypsin incubation or the careful heating of DNA solutions: (a) The distribution of the sedimentation coefficient becomes uniform after incubation with the proteolytic enzyme¹⁷. (b) The molecular weight and the mass per unit length measured by means of light-scattering becomes 'no mal', *i.e.* compatible with the values of the WATSON-CRICK double helix; whereas, from the high values observed with untreated material, parallel aggregation is deduced¹³.

Once the concept of protein-links is accepted, the assumption of a homogeneous distribution of the linking agents, instead of or together with parallel aggregation, becomes a very reasonable addition. Light-scattering measurements then must obviously be excluded from the common method of evaluation — extrapolation to zero

concentration and use of the Guinier-approximation for small scattering angles -- because for a highly crosslinked system the concept of a molecular weight and of a radius of gyration is no longer tenable. Only the mass per unit length retains its previous physical meaning. The negative intercept on the ordinate of the asymptotes always obtained by extrapolation to great scattering angles points to crosslinks¹³.

In the case of DNA, as in the case of typical polyelectrolytes, there is a very pronounced increase of the initial slopes of the curves of η_{spec}/c vs. c with decreasing ionic strength. To the sharp maxima at low ionic strength observed with polyelectrolytes correspond well developed inflexions in the case of DNA. The inflexions, according to DARSKUS *et al.*¹⁸ were to be correlated with changes of the polymer configuration as a consequence of the changed counterion distribution. Inflexions are observed at approx. 10^{-5} g DNA/ml. Suppose now a simple regular network with a distance of approx. 2μ between two crosslinks as a mean value, corresponding to a molecular weight of approx. $4 \cdot 10^6$. The network would be fully stretched then at exactly the DNA concentration of 10^{-5} g/ml. Because of inevitable shear forces, or disaggregation due to the changed counterion distribution, further diluting of the solution could lead to a partial disruption of the network which would be equivalent to the configuration change postulated by DARSKUS *et al.*¹⁸.

In the same concentration range also the pronounced self-sharpening effect observed in sedimentation experiments with DNA disappears, together with the dependence of the sedimentation coefficient on the angular velocity as reported by several authors¹⁹⁻²¹ in case of high molecular weight DNA. The last mentioned effect especially could be well interpreted on grounds of the ideas set forth in this paper: due to the crosslinked structure of the sedimenting material an additional small centrifugal potential originating from the differences of the sedimentation velocity in different parts of the cell, must be effective. It must lead to a deconcentrating effect near the boundary and to an apparent dependence of S on the angular velocity.

The anomalies which appear to be satisfactorily explained by the moderate stiff coil hypothesis of DNA in solution, could equally well be discussed with regard to a network of intrinsically flexible threads which are stretched due to the crosslinks. High flexibility indeed has to be postulated (under very different conditions, to be sure) if the high packing density of DNA in phage heads is considered. The assumption of sharp inflexions, obviously necessary in case of stiff rather than flexible threads, is rendered obsolete by current electron microscopic observations.

Fully aware that we have omitted in our discussion numerous arguments which favour the single thread hypothesis which, by the way, might be correct for DNA of viruses and microorganisms only, it has been our intention to forward arguments in favour of a network model. Despite its shortcomings, this model appears to explain many of the experimental findings in an adequate manner. Further investigations on this problem are under way.

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Biochim. Biophys. Acta, 123 (1966) 286-297

Curriculum vitae

Ich, JÖRG RICHTER, wurde als deutscher Staatsangehöriger am 21. Mai 1937 in Oldenburg i. O. geboren. Mein Vater, der Architekt Johannes Richter, fiel 1941 an der Ostfront. Meine Mutter Margarethe Richter, geb. Klopp, lebt jetzt in Darmstadt-Eberstadt. Sie ist noch heute mit 60 Jahren als Sekretärin tätig.

Nach Jahren des Umherziehens kamen wir 1948 in Blankenburg am Harz für einige Zeit zur Ruhe. Ich besuchte dort mit meinen Geschwistern die Oberschule. Direkt nach meiner bestandenen Reifeprüfung verliessen wir 1955 – alle Habe zurücklassend – die DDR und gingen nach Darmstadt. Dort bestand ich 1957 die Ergänzungsprüfung zur DDR-Maturität und begann im Herbst des gleichen Jahres mit dem Studium an der Fakultät für Mathematik und Physik der Technischen Hochschule in Darmstadt.

Mit abgelegtem Vorexamen kam ich 1960 – ursprünglich für nur zwei Auslandssemester – nach Basel. Einer neuerwachten Neigung folgend, beschäftigte ich mich in dieser Zeit ausser mit Physik intensiv mit deutscher Literatur. Ich sah jedoch bald ein, dass ich zwei so verschiedene Studien nicht parallel betreiben konnte. Um meinen Entschluss, in Basel zu bleiben, verwirklichen zu können, musste ich selbständig werden. Deshalb versuchte ich mich als Lehrer in einem Privatinstitut in Oberägeri, Kt. Zug. Um die Kontinuität meines Studiums wenigstens formal zu wahren, besuchte ich an einem Tage in der Woche Vorlesungen in Zürich.

Im Frühjahr 1962 heiratete ich Fräulein Françoise Schubert aus Uttwil im Thurgau und kehrte mit ihr nach Basel zurück. Ich setzte meine Studien in physikalischer Richtung fort und betätigte mich daneben bis 1964 als Lehrer am hiesigen Mädchen-gymnasium. Im Sommer 1963 begann ich unter der Leitung von Herrn Prof. M. Thürkauf eine Arbeit über die Konfiguration von Desoxyribonukleinsäure in Lösung, wovon ich ein Teilergebnis im Frühjahr dieses Jahres als Dissertation vorlegte. Zur Zeit bin ich als wissenschaftlicher Mitarbeiter der Schweizerischen Kommission für Atomwissenschaft am Physikalisch-chemischen Institut tätig.

Während meiner Studienzeit besuchte ich die Vorlesungen, Übungen, Seminare und Praktika der Herren Professoren und Privatdozenten:

In Darmstadt: Börsch-Supan, v. Buttler, Graf, Koehler, Kohlschütter, König, Menzel, Messmer, Schlechta, Schmieden, Schnabl, Schnell, Stocker, Stöckmann, Walther, Witte.

In Zürich: phil. II: B. L. v. d. Waerden; phil. I: A. Joost, J. Roos.

In Basel: phil. II: K. Alder, P. Baertschi, E. Baldinger, R. Conzelmann, P. Diehl, F. Grün, P. Huber, W. Kuhn, M. Thürkauf, P. Scherrer, E. Schubarth; phil. I: W. Kohlschmidt, E. Müller, W. Muschg, H. Rupp, L. Wiesmann.

Basel, im Mai 1965

JÖRG JOHANNES RICHTER

